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Short-term exposure to a synthetic estrogen disrupts mating dynamics in a pipefish

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ABSTRACT

Sexual selection is responsible for the evolution of some of the most elaborate traits occurring in nature, many of which play a vital role in competition over access to mates and individual reproductive fitness. Because expression of these traits is typically regulated by sex-steroids there is a significant potential for their expression to be affected by the presence of certain pollutants, such as endocrine disrupting compounds. Endocrine disruptors have been shown to alter primary sexual traits and impact reproduction, but few studies have investigated how these compounds affect secondary sexual trait expression and how that may, in turn, impact mating dynamics. In this study we examine how short-term exposure to a synthetic estrogen impacts secondary sexual trait expression and mating dynamics in the Gulf pipefish, a species displaying sex-role reversal. Our results show that only 10 days of exposure to 17α-ethinylestradiol results in adult male pipefish developing female-like secondary sexual traits. While these males are capable of reproduction, females discriminate against exposed males in mate choice trials. In natural populations, this type of discrimination would reduce male mating opportunities, thus potentially reducing their long-term reproductive success. Importantly, the effects of these compounds on mating dynamics and mating opportunity would not be observed using the current standard methods of assessing environmental contamination. However, disrupting these processes could have profound effects on the viability of exposed populations.

Introduction

Sexual selection is a major evolutionary mechanism that often results in the evolution of secondary sex traits, which play a significant role in competition for access to mates. Mate choice mechanisms often lead to the development of elaborate ornaments that are used by individuals to assess mate quality, and experimental alterations of these traits can significantly impact mating success. While expression of a secondary sexual trait is normally limited to one sex, the genes influencing their development are usually present in the genomes of both sexes (Andersson, 1994). Sex-specific expression of these traits is often the result of the relevant genes being under the control of sex-steroid hormones, testosterone and estrogen (Andersson, 1994; Folstad and Karter, 1992; Parker et al., 2002; McGraw, 2006; McGraw et al., 2006). Because slight changes in hormone levels may affect secondary sexual trait expression without severely affecting primary sex traits, secondary sex traits may be more sensitive to small levels of environmental pollution, particularly when the pollutants are endocrine disrupting compounds.

Endocrine disruptors are compounds that interfere with normal hormone function and have been shown to greatly impact population viability by interfering with reproduction (Kidd et al., 2007). In addition, studies have shown that exposure to environmental pollutants can affect the development of secondary sexual traits, causing them to be suppressed (Arellano-Aguilar and Garcia, 2008) or expressed in the opposite sex (Ueda et al., 2005; Larsen et al., 2008). Most of the research concerning the impact of endocrine disruptors on population health has focused on their effects on primary sexual organs, particularly the gonads (Allen et al., 1999; Hill and Janz, 2003; Weber et al., 2003; Moncaut et al., 2003; Vandenbergh et al., 2003; Palace et al., 2006; Pettersson et al., 2006; Brown et al., 2008), and reproductive ability (Hill and Janz, 2003; Maunder et al., 2007; Peters et al., 2007; Schäfers et al., 2007; Hashimoto et al., 2009). While the number of studies concerning the effect of these compounds on pre-copulatory mating behavior has increased (Bell, 2001; Bjerselius et al., 2001; Oshima et al., 2003; Robinson et al., 2003; Crews et al., 2007; Saaristo et al., 2009a,b), only a few studies have assessed how endocrine disruptors impact secondary sexual trait expression (Ueda et al., 2005; Arellano-Aguilar and Garcia, 2008; Larsen et al., 2008) and how that may, in turn, affect mate choice mechanisms (Arellano-Aguilar and Garcia, 2008). This situation is particularly surprising since the ability of individuals to maintain secondary sexual traits significantly influences their mating success. One potential reason for this gap in knowledge is that many of the toxicological model species

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do not maintain obvious secondary sexual traits involved in mate choice. However, mate choice and sexual selection are key components of reproduction and can significantly influence population viability, so there is a critical need to understand the effects of endocrine disruptors on these evolutionary processes. In this study, we examined how short-term exposure to environmentally relevant concentrations of a synthetic estrogen affected multiple aspects of reproductive fitness, including mating opportunity, mating success and reproductive success, in a sex-role reversed pipefish characterized by strong mating preferences and sexual selection.

One endocrine disruptor that has received a large amount of attention is 17α-ethinylestradiol (EE2). EE2 is a synthetic estrogen that is one of the major components of oral contraceptives. A high resistance of EE2 to degradation in the human body is one feature that makes it useful in contraceptives. However, this characteristic also allows EE2 to pass into the environment through domestic wastewater (Hill and Janz, 2003; Lintelmann et al., 2003), and high concentrations of EE2 have been found in wastewater effluent and rivers within the United States and Europe (Kolpin et al., 2002; Ying et al., 2002; Clouzot et al., 2008). Of the vast array of potential endocrine disrupting chemicals, EE2 is one of the most troubling because it binds to estrogen receptors with high affinity and, compared to other estrogenic compounds, is relatively stable in the environment. The amount of EE2 in wastewater treatment plant effluent is variable depending upon season and effectiveness of water treatment but can range from 0.1 to 10 ng/L (Kolpin et al., 2002; Clouzot et al., 2008; Vajda et al., 2008). Surface waters around treatment plants commonly show EE2 concentrations of 0–5 ng/L (Clouzot et al., 2008; Vajda et al., 2008). However, a study by Kolpin et al. (2002), which evaluated EE2 concentrations in 139 U.S. contaminated rivers, found maximum concentrations of EE2 to be 820 ng/L, and concentrations approaching 35 ng/L in Europe have been reported (Pojana et al., 2007). These levels are of significant concern considering that exposure to 0.5 ng/L EE2 can induce vitellogenin production in males of some species (Nash et al., 2004), and a whole-lake experiment showed that 5–6 ng/L EE2 caused a population of fathead minnows to collapse after only two seasons of exposure (Kidd et al., 2007). This sort of population collapse could result from effects of EE2 on gonads or other primary sexual traits (Bell, 2001; Bjerselius et al., 2001; Robinson et al., 2003; Balch et al., 2004; Nash et al., 2004; Maunder et al., 2007) or by disrupting mating patterns so severely that population viability is reduced. To date, only a few studies have evaluated how EE2 impacts mechanisms of sexual selection and mate choice (Arellano-Aguilar and Garcia, 2008; Coe et al., 2008; Saaristo et al., 2009a,b), so our goal was to study EE2 exposure in a system characterized by ritualistic courtship behaviors and strong sexual selection.

The Gulf pipefish, *Syngnathus scovelli*, serves as an interesting model system for this type of study for a number of reasons. First, this species is sex-role reversed, in that sexual selection acts more strongly on females than on males (Jones and Avise, 1997; Jones et al., 2001). Theoretically, sex-role reversal can evolve due to a number of factors such as higher potential reproductive rates in females (Clutton-Brock and Vincent, 1991; Clutton-Brock and Parker, 1992; Ahnesjö et al., 2001), female-biased operational sex ratios (Emlen and Oring, 1977; Kvarnemo and Ahnesjö, 1996), higher investment in parental care by males relative to females (Trivers, 1972), or costs of breeding (Kokko and Monaghan, 2001; Simmons and Kvarnemo, 2006). Regardless of the reason for this reversal, availability of males limits female reproductive fitness and thus leads to an increase in competition between females over access to males. In the Gulf pipefish, females transfer unfertilized eggs into a specialized brood pouch on the ventral surface of the male during mating. The male fertilizes the eggs by releasing sperm into the pouch, and then carries the developing embryos for approximately two weeks until they are born as independent juveniles. Gulf pipefish are polyandrous in that males typically receive eggs from one female per "male pregnancy", but females are capable of filling multiple pouches (Jones and Avise, 1997; Jones et al., 2001). Consequently, males are a limiting resource for reproduction, and females compete for access to males, resulting in strong sexual selection on females and weak sexual selection on males (Jones and Avise, 1997; Jones et al., 2001), a reversal of the usual direction of sexual selection seen in most vertebrates, including teleost fishes. As a result of this selection on females, secondary sexual traits have evolved in females rather than males. Adult females are larger than males and possess a number of sex-specific characteristics that develop during maturation, such as a deeply keeled abdomen, intense silvery-blue lateral stripes, a large dorsal fin, and striking temporary breeding coloration. Interestingly, while many studies examining hormonal regulation of secondary sex traits in species with typical sex roles have found that testosterone concentrations influence ornament expression, a study by Ueda et al. (2005) found that male Gulf pipefish exposed to high concentrations of EE2 (100 ng/L) developed the silvery-blue lateral strips that are normally only present in females. These data suggest that expression of secondary sexual traits in this species may be affected by estrogen exposure, which consequently may impact mating dynamics.

Examining how estrogen exposure impacts reproductive success in the Gulf pipefish is also interesting in light of the fact that pipefish may not possess the "typical" sex steroid profiles found in many other teleosts. Sex steroid profiles from two *Syngnathus* species (*Syngnathus acus* and *Syngnathus typhle*) have shown that both breeding and brooding males have higher plasma testosterone (T) levels compared to 11-ketotestosterone (KT) (Mayer et al., 1993), which is the opposite of what is found for many other teleosts. In addition, these males also contain measurable levels of 17ß-estradiol (E2) during both the breeding and brooding period (Mayer et al., 1993). Thus, these differences in steroid profiles may result in differences in the effect of endocrine disruptors on pipefish mating and reproductive success when compared to studies from other teleost species. The questions we aim to address in this study are: (1) Do environmentally relevant concentrations of EE2 produce males that are feminized with respect to secondary sexual traits? And (2) are these effects of sufficient magnitude to disrupt mating patterns and sexual selection?

We addressed these questions by examining the effects of EE2 in several different experiments, including (1) a test of the effects of EE2 exposure on male morphology, (2) mate choice trials involving males and females choosing between exposed and unexposed individuals of the opposite sex, and (3) measurement of the effects of EE2 exposure on the ability of males to mate and to carry their pregnancy to term. Our work provides one of the most comprehensive studies of the effects of an endocrine disrupting compound on reproductive behavior and the only investigation of the effects of such contaminants on a sex-role reversed organism.

Methods

Pipefish collection and maintenance

A hand seine was used to collect pipefish from submerged vegetation at the northern end of Mobile Bay in Meaher State Park, Baldwin County, Alabama, USA (30°66624, 87°92731). The breeding period of the Gulf pipefish in Mobile Bay typically lasts from May to December, with a peak between August and October (Bolland and Boettcher, 2005). For this study, pipefish were collected between June and November in the years 2006 through 2008 and all exposure experiments were performed between the months of July and November, to ensure that individuals were reproductively viable. Males and females were housed separately in 30 L aquaria connected to a recirculating filtration system. Pipefish were fed a diet of *Artemia* nauplii twice daily and their diets were supplemented with live copepods biweekly. All pregnant males were allowed to give birth prior to exposure to control for male reproductive state. All

procedures performed for this study were conducted in accordance with both the University of South Alabama and Texas A&M University IACUC regulations.

Mate choice of exposed versus non-exposed individuals

Exposure

Control (0 ng/L EE2), 1 ng/L EE2 and 100 ng/L EE2 exposure tanks were maintained on separate recirculating systems. These three concentrations were chosen to simulate an EE2-free environment (Control), an environment potentially down-stream from a waste-water treatment plant (1 ng/L EE2) and a highly contaminated area (100 ng/L EE2). The control system consisted of fifteen 30 L aquaria, while the two treatment systems consisted of nine 30 L aquaria each. All tanks were divided into two 15 L compartments by a perforated barrier. The control system was initially spiked with 510 μL of 95% ethanol, the solvent for the EE2 solution, to account for the 450 L of water within the tanks and a 60 L sump for a total of 510 L. Treatment systems were initially spiked with 330 μL of either 1 mg/L EE2 (for 1 ng/L system) or 100 mg/L EE2 (for 100 ng/L system) to account for the 270 L of water within the tanks and 60 L sumps for a total of 330 L per treatment system. Prior to exposure, same sex individuals (for both males and females) were size and color matched and then paired. Size and color matching was performed in order to control for other confounding factors that may influence mate choice. One of the individuals of the pair was assigned to a 15-L compartment in the control system, while the other individual was randomly assigned to a 15-L compartment in either the 1 ng/L or 100 ng/L EE2 system. At any given point during the experiment, tanks within each system contained at most two individuals (i.e., one individual per 15 L compartment). Males and female were housed on separate shelves within a system so that biases in visual cues among individuals would not occur during the exposure time. All individuals were exposed for a total of 10 days. We performed 2% water changes (including appropriate amounts of either 95% ethanol or EE2 in 95% ethanol for control and exposure tanks, respectively) and cleaned the tanks daily. While the actual concentration of EE2 within these systems was not measured, preliminary studies indicated that this regimen of daily water changes maintained relatively constant EE2 concentrations over time.

Body morphology

Before exposure, we measured the standard length of each male. We also used a Nikon Coolpix 5000 digital camera to photograph each male in lateral view against a size standard. From the photographs, we used the computer program *Image J* (NIH, Bethesda, MD, www.rsbweb.nih.gov/ij/) to measure maximum body depth. We then calculated the depth:length ratio by dividing the maximum body depth by the standard length. We measured each individual a second time at the conclusion of each exposure period and mate choice trial.

To quantify the effects of EE2 on male coloration, the images taken of the lateral view of each male both prior to and after exposure (see above) were analyzed using *Image J*. The red color was removed from all pictures in order to obtain images that provided the best color differentiation to detect changes in the banding pattern. The maximum and mean color intensity was then obtained for an area approximately 1.3 cm long and 0.02 cm high along the lateral side of the male, where the iridescent stripes develop. The maximum color intensity, defined as the value of the brightest pixel within that area, was then divided by the mean color intensity of the area. Because the areas within the iridescent bands produce the highest color intensity, we were able to assess whether areas of iridescence developed in exposed males by evaluating the relationship between maximum color intensity and mean body color intensity along the area where these iridescent stripes develop.

Mate preference trials

All preference trials were performed in the morning 10 minutes after the beginning of the 12 hour daylight cycle. We used either a Cannon Optura 500 MiniDV or a Sony HDV 1080i MiniDV video camera to videotape all mate choice trials for 1 hour. Behaviors were analyzed using JWatcher version 1.0 animal behavior software (University of California Los Angeles, Los Angeles, CA: http://www.jwatcher.ucla.edu/).

Male preference

The effect of each treatment on male preference was determined by using a mate-choice design in which the focal male had the ability to assess control and exposed females but the females could not interact with each other (Fig. 1). Each mate-choice tank was divided lengthwise by a transparent barrier. One of the resulting long chambers was divided in half by an opaque divider orthogonal to the transparent barrier. Two females were placed in the tank, with an exposed female in one small chamber (25 W × 28 H × 12.5 D cm) and a control female in the other small chamber (25 W × 28 H × 12.5 D cm). The male was placed into the longer compartment (50 W × 28 H × 12.5 D cm), from which he could see into both the smaller compartments. The females, separated by the opaque divider, could not see each other but they could observe the male. The proportions of time the male spent performing the following behaviors were examined: (1) swimming near the divider close to a female (defined as the male swimming in a zigzag pattern up and down near the divider), and (2) dancing (defined as an up and down bobbing type movement with the body in a strict vertical position). In addition, the number of twitches (defined as abrupt shaking of the individual lasting for less than a second) performed by the focal male toward each female was recorded. For a quantitative measure of mate choice, we lumped the male behaviors of swimming near the divider and dancing into a more inclusive category termed "male response time." We also recorded the proportion of time each focal male spent on the side of the tank with each female. Finally, we recorded and analyzed the behavior of each female (see below) to test for effects of exposure to EE2 on female courtship behavior.

Female preference

The effect of each treatment on female mate choice was determined by evaluating the relative proportion of time females spent actively courting each male and the number of twitches females

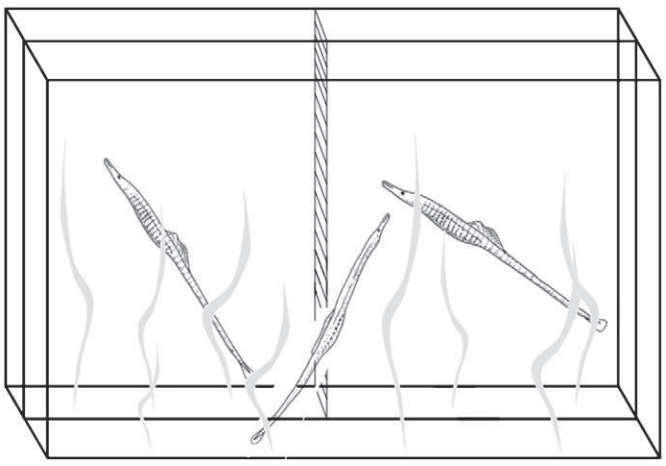

Fig. 1. Diagram of mate choice tank. For male choice trials the focal male was placed in the front compartment while choice females were each placed within the separate back compartments. The focal male was separated from choice females by a clear perforated plexi-glass divider to allow for both visual and chemical cues to be detected. Choice females were separated by an opaque plexi-glass divider to prevent visual contact between the individuals. For female choice trials the mate choice design was similar except that the focal female was placed within the front compartment while the choice males were each placed in the separate back compartments.

performed toward each male. We used the same experimental setup as in the male-choice trials (see above). Thus, each trial involved a focal female assessing two males, one of which had been exposed to EE2 and one of which was a control male. The female was able to visually assess both males and males were able to assess the focal female; however, males were separated from one another by an opaque divider. The proportions of time the female spent performing the following behaviors toward each male were recorded: (1) color display (defined as the female displaying a darkened, erect dorsal fin and high contrast transverse stripes along the body), (2) posing (defined as the female moving sharply into a vertical position and assuming a distinct posture with the abdomen protruding), and (3) dancing (defined above). We also recorded the number of twitches (defined above) performed toward each male. In *S. scovelli*, the female color display appears to be a static mating behavior, possibly indicating female receptivity, as this behavior also occurs in the absence of males. However, posing and dancing are behaviors that females actively perform toward other individuals. Thus, the proportion of time females spent posing and dancing were grouped to represent active female courtship behavior. Finally, we recorded the proportion of time focal females spent on the side of the tank with each male.

Mating success and reproductive success

Mating and reproductive success were evaluated two different ways. For years 2006 and 2007, after each mating trial, control and exposed males were placed individually in 15 L compartments to which a female that had not been involved in a mating trial was added. We checked males each morning to determine whether or not they were pregnant. Once impregnation occurred, we removed the female. Pregnant males were allowed to brood their offspring, and we recorded the amount of time that elapsed from impregnation until birth.

In 2008, we exposed ten non-pregnant males each to control water (spiked with the appropriate amount of 95% ethanol), 1 ng/L EE2 water, and 100 ng/L EE2 water. After ten days, males were moved to clean tanks containing females. Given that females can impregnate multiple males (Jones et al., 2001), we housed two females with five males in each tank. Males were checked twice daily for pregnancy. Pregnant males were removed and maintained individually in 15 L aquarium compartments, where we monitored the progression of their pregnancies. After three days, females from each tank were rotated to ensure that any male's failure to become pregnant was not attributable to the females with which he was housed. Pregnant males nearing parturition, which occurs after 12–15 days of brooding, were moved to individual 2 L birthing chambers. For each male, we counted the number of offspring born as a measure of his reproductive success.

Statistics

For our statistical analysis, we first transformed our data by using arcsine (square root) transformations on all proportion data and square-root transformations on the number of twitches females performed to each male, the number of twitches males performed to each female and the maximum/mean color intensity of males. A repeated measure analysis of variance (ANOVA) showed that male depth:length ratios significantly differed between pre-and post-exposure periods and that a significant interaction existed between EE2 concentration and change in maximum/mean color intensity. In order to determine how each concentration affected these two traits, we compared male body depth:length ratio and maximum/mean color intensity between exposed and unexposed males prior to and after exposure by using paired *t*-tests. For the behavioral analysis, we initially used a multivariate repeated-measures ANOVA to examine whether focal individuals behaved differently to control and exposed individuals and whether these differences were affected by EE2 concentration. To examine which specific behaviors differed in response to control and treated individuals, *t*-tests of the mean were used to determine if the proportion of time focal females spent with each male, the relative proportion of time focal females actively courted males, the proportion of time focal males spent with each female and the relative proportion of time focal males responded to a female differed from a random expectation of 0.5. In the male mate choice experiments (two females with one male per trial), we used paired *t*-tests to compare control and experimental females with respect to: (1) number of twitches performed, (2) extent of color display, and (3) amount of active courtship. In this case, paired *t*-tests were used since females within a trial were responding to the same focal male and were therefore not independent. A Wilcoxon signed ranks test was used to determine if the difference in number of twitches control and exposed females performed in male choice tests differed from 0. A chi-square analysis of a 3×2 contingency table was used to examine whether or not a male's ability to become pregnant was dependent upon exposure. Finally, we used an ANOVA to compare the mean number of offspring produced by males subjected to different treatments.

Results

With respect to morphology, we found that short-term exposure to EE2 had a significant effect on male phenotype. After only 10 days of exposure, males from exposure treatments showed a significant change in body depth:length ratios (ANOVA: $F_{1,\ 35}=58.57$, $p<0.001$) with the body depth:length ratio of exposed males becoming significantly greater than their control partners (Table 1). This is particularly interesting considering in natural populations reproductively active female pipefish have a larger body depth (measured at the thickest part of the abdomen) than males. In addition to body shape, exposure to EE2 may also effect male body coloration. While there was no overall effect of exposure on changes in color intensity (ANOVA: $F_{1,\ 39}=2.64$, $p=0.07$), a significant interaction between EE2 concentration and changes in color intensity was observed (ANOVA; treatment*color intensity: $F_{2,\ 39}=6.68$, $p=0.003$). Exposure to 100 ng/L EE2 caused males to develop iridescent lateral stripes, which are normally only found in female pipefish (Fig. 2) and differences in the maximum/mean color intensity along the banding area were observed after exposure (Table 1). Some of the 1 ng/L EE2 exposed males also developed these female-like iridescent stripes, although the expression was not as pronounced as in males exposed to the higher concentration (Fig. 2). In addition, while no significant difference in maximum/mean color intensity was observed between control and 1 ng/L EE2 males after exposure, 1 ng/L EE2 exposed males tended to have areas within the banding area that were brighter compared to control males (Table 1).

Given that EE2 exposure affects male morphology, the next question was whether or not such changes potentially impact mating dynamics in this species. Our results clearly show that male attractiveness is affected even by low doses of EE2, whereas female mating behavior and attractiveness are largely unaltered. In female choice trials, where focal females were able to choose between control and exposed males, multivariate repeated-measures ANOVA showed that focal female behavior significantly differed in response to control and exposed males ($F_{3,\ 84}=32.38$, $p<0.001$) and that the concentration of EE2 to which males were exposed had a significant effect on female behavior ($F_{1,\ 84}=11.59$, $p<0.001$). More specifically, we found that focal females spent more time actively courting (posing and dancing; Table 2) and performed more twitches (Table 2) toward control males compared to males exposed to either 1 ng/L or 100 ng/L of EE2. Females did not differ in the proportion of time they spent in their color display between control and treatment males (Table 2). However, female color display is likely a general indicator of female receptivity, and not necessarily indicative of choice, since females will

Table 1
The effects of EE2 exposure on male body morphology.

	Control male	1 ng/L EE2 male	*N*	Paired *t*-test	*p*-Value	Control male	100 ng/L EE2 male	*N*	Paired *t*-test	*p*-Value
Pre BD:length ratio	0.039 ± 0.002	0.038 ± 0.002	9	$\|t\|_8 = 0.82$	0.38	0.042 ± 0.001	0.042 ± 0.001	10	$\|t\|_9 = 0.189$	0.85
Post BD:length ratio	0.038 ± 0.004	0.041 ± 0.003	9	$\|t\|_8 = 3.06$	0.01	0.042 ± 0.001	0.050 ± 0.001	10	$\|t\|_9 = 28.28$	0.0003
Pre max/mean CI	2.33 ± 0.14	2.37 ± 1.20	11	$\|t\|_{10} = 0.13$	0.9	2.56 ± 0.21	2.47 ± 0.24	10	$\|t\|_9 = 0.25$	0.78
Post max/mean CI	2.01 ± 0.15	2.68 ± 0.35	11	$\|t\|_{10} = 2.01$	0.07	2.30 ± 0.19	3.28 ± 0.28	10	$\|t\|_9 = 2.88$	0.02

Body depth (BD):length was calculated by dividing maximum body depth by standard length. Maximum/mean color intensity (CI) is the maximum color intensity divided by the mean color intensity of a 1.3 cm long × 0.2 cm high area along the lateral side of the males where the iridescent stripes develop. Initially, repeated measure ANOVA showed that exposure affected both body depth:length ratios and maximum/mean color intensity (see text). Paired *t*-test were then used to compare BD:length and maximum/mean CI between control and experimental males before (Pre) and after exposure (Post) for both dosages. All data are reported as mean ± S.E of untransformed data.

invoke their color display even in the absence of males. Therefore, based upon these results, EE2 exposure clearly affects male mating opportunity by altering their attractiveness to females, as might be expected from the feminizing effects of this compound.

In contrast to our results for males, we found no detectable effects of EE2 exposure on female attractiveness. Male pipefish did not significantly differ in their response between control females and EE2 exposed females from either the 1 ng/L or 100 ng/L EE2 treatments (Table 3). Likewise, female mating behavior did not differ significantly between the two treatments (Table 3). In male mate choice tests with non-exposed females, female behavior appears to be a major factor influencing male response time (unpublished data), and thus the lack of an effect of EE2 on female courtship behavior likely explains why exposure did not affect male mate preference.

In our final experiment, we addressed the question of whether or not exposure to EE2 affects mating ability and male pregnancy. When females were given no choice (see methods), they mated as successfully with males exposed to 1 ng/L EE2 ($N = 18$; number impregnated = 9) as with control males ($N = 33$, number impregnated = 19). However, at some point exposure to EE2 limits the efficacy of mating, as we found that males exposed to 100 ng/L of EE2 were significantly less likely to become pregnant ($N = 19$; number impregnated = 4) than control males (for all three treatments, contingency-$\chi^2 = 6.66$; $p = 0.04$). Of the 100 ng/L EE2 males that mated, these mating events did not occur until at least four days after removal from the exposure tanks, suggesting that there may be a lag time after removal from treated water during which males are not able to mate. For those males that became pregnant, exposure to EE2 did not affect the number of offspring in their broods (Control: 13.9 ± 4.4 [mean ± S.E.], $n = 8$; 1 ng/L: 16.2 ± 3.8, $n = 8$; 100 ng/L: 16.3 ± 5.2, $n = 4$, $F_{2,19} = 0.10$, $p = 0.9$). Development of the offspring also appeared to be unaffected, as males that mated within 10 days after removal from treated water produced successful broods with completely developed juveniles, regardless of treatment. It is

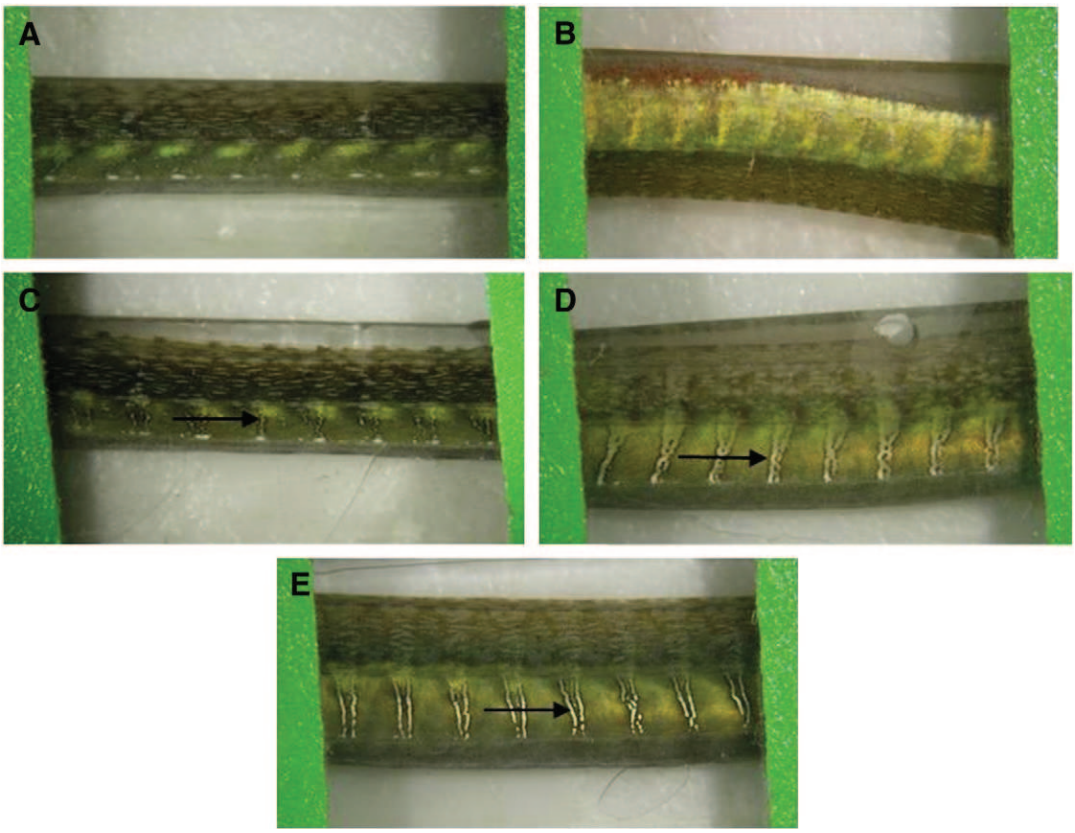

Fig. 2. Induction of secondary sexual traits in male pipefish after exposure to EE2. (A) Control male prior to exposure, (B) Control male after 10 days of exposure to 0 ng/L EE2, (C) Treatment male after 10 days of exposure to 1 ng/L EE2, (D) Treatment male after 10 days of exposure to 100 ng/L EE2, (E) Non-exposed female. The black arrows point to the iridescent stripes, a secondary sex trait normally confined to females. The green bars at the ends of each photograph indicate a spacing of 1.5 cm. This figure shows that exposure of males to EE2, especially the 100 ng/L concentration, induces the development of female-like secondary sexual traits. Thus, EE2 feminizes the males with respect to external morphology.

Table 2
Effects of EE2 exposure on female mate choice.

	Control vs 1 ng/L EE_2 Male					Control vs 100 ng/L EE_2 Male				
Female Behavior	Control Male	1 ng/L Male	*N*	*t*-Test	*p*-Value	Control male	100 ng/L male	*N*	*t*-Test	*p*-Value
Prop of time with male	0.58 ± 0.06	0.42 ± 0.06	11	$\|t\|_{10} = 1.38$	0.2	0.63 ± 0.06	0.37 ± 0.06	12	$\|t\|_{11} = 2.02$	0.07
Rel prop of time in color display	0.57 ± 0.06	0.43 ± 0.06	11	$\|t\|_{10} = 1.19$	0.26	0.60 ± 0.08	0.40 ± 0.08	12	$\|t\|_{11} = 1.38$	0.2
Rel prop of time active court male	0.65 ± 0.06	0.35 ± 0.06	11	$\|t\|_{10} = 2.49$	0.03	0.80 ± 0.05	0.20 ± 0.05	12	$\|t\|_{11} = 5.04$	0.0004
Number of twitches to males	12.1 ± 3.1	3.7 ± 1.2	11	$\|t\|_{10} = 3.03$	0.01	7.4 ± 1.7	1.3 ± 0.5	12	$\|t\|_{11} = 4.46$	0.001

The experiment consisted of a female choosing between two males, one of which had been exposed to EE2 and one of which had not. Female behaviors included the proportion of time female spent on the same side of the tank as each male, relative proportion of time female was in color display in front of each male, relative proportion of time female actively courted each male and the number of twitches females performed to each male. Multivariate repeated measures ANOVA showed that female behavior differed toward control and exposed males and the concentration to which males were exposed also influenced female behavior (see text). To evaluate which specific female behaviors differed, *t*-tests of the mean were used to determine if the proportion of female behavior toward a specific male differed from an expected value of 0.05. All data are reported as mean ± S.E. of untransformed data.

important to note, however, that during the period of brood development all males were housed in EE2-free water.

Discussion

Overall, our results lead to two important conclusions regarding the impact of estrogen mimics on secondary sexual trait expression and mating dynamics in the Gulf pipefish. First, our data suggests that expression of the iridescent bars present in female *S. scovelli* appear to be affected by estrogenic compounds. Males exposed to EE2 developed iridescent bars and expression appears dose dependent. Second, it suggests that short-term exposure to even low levels of EE2 can significantly impact male reproductive fitness. Male attractiveness was significantly affected by exposure to 1 ng/L EE2, even though these males could still become pregnant and successfully carry broods to term. The results were even more pronounced when males were exposed to higher doses of EE2. For example, males exposed to 100 ng/L of EE2 were not only less likely to attract females than control males, but they also experienced difficulty becoming pregnant. These effects occurred after only 10 days of exposure to EE2, and some of the morphological changes persisted well after the males were removed from the contaminated water (at least 22 days).

While many studies have addressed how endocrine disruptors impact reproductive success, very few studies have addressed how these compounds impact secondary sexual trait expression, even though, in many cases, trait expression and reproductive success are correlated. Of the secondary sexual traits that have been studied, androgens (namely testosterone) have been shown to influence trait expression in both males and females (Rand, 1992; Eens et al., 2000; Parker et al., 2002; McGraw, 2006). However, in the present study, we found that exposure to EE2 significantly impacted trait expression in male pipefish, with exposed males developing secondary sexual traits normally restricted to females. While previous studies have shown that some male pipefish species (*S. acus* and *S. typhle*) naturally maintain detectable levels of 17-ß estradiol (E2) during the breeding and brooding period (Mayer et al., 1993), neither males nor females of these species possess the permanent iridescent stripes that are present in *S. scovelli* females and induced with EE2 exposure in males. In *S. scovelli* females, the initial expression of these stripes occurs during female maturation, also suggesting that estrogen levels may play a role in their development. Thus, this observation suggests that, if male *S. scovelli* follow the same sex steroid profile as other *Syngnathus* species, a minimum concentration of estrogen may be needed to actually induce expression of these iridescent stripes. However, whether these traits are solely estrogen dependent is unclear since females will continue to express this trait even during the non-breeding cycle and the stripes in males persisted after removal from treated water.

Given that exposure to EE2 significantly impacted secondary sexual trait expression in males, the next question was whether or not this effect influenced mate choice mechanisms in this species. Clearly, our data show that exposure to EE2 significantly impacted male mating opportunity, since females discriminated against exposed males from both 1 ng/L and 100 ng/L EE2 treatments. So, why does EE2 decrease male attractiveness? One possible hypothesis is that the induction of secondary sexual traits in males may mimic female-specific visual cues, causing females to incorrectly identify exposed males as other females. Alternatively, EE2 exposure may cause males to release chemicals that contain components that typically are released only by females. However, female pipefish do not readily discriminate between males and females based on chemical cues alone (Ratterman et al., 2009), suggesting that our results are more likely related to visual cues. Males exposed to high doses of EE2 could experience high mating failure rates for a number of reasons. First, as

Table 3
The effects of EE2 exposure on male mate choice and female courtship behavior.

	Control vs 1 ng/L EE_2 Male					Control vs 100 ng/L EE_2 Male				
Male Behavior	Control Female	1 ng/L Female	*N*	t-test	*p*-Value	Control Female	100 ng/L Female	*N*	*t*-Test	*p*-Value
Prop of time with female	0.48 ± 0.12	0.52 ± 0.12	10	$\|t\|_9 = 0.27$	0.8	0.40 ± 0.09	0.60 ± 0.09	9	$\|t\|_8 = 0.98$	0.36
Rel prop of time responding to female	0.51 ± 0.13	0.49 ± 0.13	10	$\|t\|_9 = 0.11$	0.92	0.49 ± 0.14	0.51 ± 0.14	8	$\|t\|_7 = 0.03$	0.98
Number of twitches to females	1.3 ± 0.06	4.3 ± 1.4	7	$\|t\|_6 = 1.36$	0.22	6.1 ± 3.2	1.2 ± 0.7	6	$\|t\|_5 = 1.05$	0.33
Female behavior	Control female	1 ng/L female	*N*	Paired test	*p*-Value	Control Female	100 ng/L Female	*N*	Paired test	*p*-Value
Prop of time in color display	0.22 ± 0.12	0.34 ± 0.12	8	$\|t\|_7 = 0.65$	0.54	0.25 ± 0.09	0.11 ± 0.06	9	$\|t\|_8 = 1.04$	0.33
Prop of time actively courting	0.06 ± 0.09	0.15 ± 0.13	8	$\|t\|_7 = 1.20$	0.27	0.05 ± .03	0.01 ± 0.009	9	$\|t\|_8 = 0.86$	0.41
Number of female twitches	2.0 ± 1.4	6.2 ± 3.6	9	$\|z\| = 0.841$	0.4	6.6 ± 3.7	1.3 ± 1.2	9	$\|z\| = 1.08$	0.28

These data are from an experiment in which a single focal male could choose between two females, one of which had been exposed to EE2 and one of which had not. We measured both male and female behavior in these experiments. Male behavior included the proportion of time males spent on the same side of the tank as female, the relative proportion of time males spent responding to each female and the number of twitches males preformed to each female. Female courtship behaviors included the proportion of time females were in color display, the proportion of time females actively courted males and the number of twitches performed by females. Transformation of the number of twitches performed by females was unable to satisfy the assumption of normality, so a Wilcoxon signed rank test was performed. All data are reported as mean ± S.E. of untransformed data.

was just described, females may avoid mating attempts with males exposed to 100 ng/L EE2 because of the physical similarities between exposed males and female pipefish. Second, exposure to high levels of EE2 also appears to affect the male's brood pouch, where eggs are received, fertilized and develop. Boisseau (1967) found that the brood pouch of male seahorses (*Hippocampus sp.*) is under testicular control and that male castration significantly retards pouch development. This effect can be reversed by testosterone injections. The brood pouches of male Gulf pipefish, which are close relatives of seahorses, are likely under similar hormonal regulation. Males exposed to high concentrations of EE2 had brood pouches that appeared thin and resembled the pouches of non-reproductive males. Since testosterone is known to be involved in maintaining the brood pouch, it is likely that EE2 exposure interferes with testosterone levels, preventing the male brood pouch from remaining in a reproductive state. Finally, plasma vitellogenin levels of male pipefish exposed to 100 ng/L EE2 have been shown to be 10 times higher than those of field collected females and exposure is known to affect male gonadal tissue (Ueda et al., 2005), which would also impact the reproductive ability of males exposed to this high concentration. After the male is removed from treated water, it may take a few days to completely eliminate EE2 from the body and allow the brood pouch and testes to become functional again, possibly explaining why males in the high EE2 exposure treatment were unable to mate for four days after removal from the exposure treatment. While our study solely focused on how EE2 exposure prior to pregnancy impacted male reproductive success, in natural populations pregnant males also would be exposed. Thus, future studies should not only examine how exposure impacts adult reproductive fitness, but should also examine whether continuous exposure to low doses of estrogenic compounds impacts offspring health and viability.

While exposure to EE2 clearly affects mating dynamics in this species, one additional troubling implication of our observations is that the effects of this compound may not be restricted to contaminated sites. Some of the female secondary sexual characteristics that are induced in males upon exposure are maintained for some time after removal from treated water. We found that males continued to express the iridescent bars that are normally found on females for at least 19–22 days after removal from treated water (Fig. 3). After this point males were sacrificed, so the amount of time exposed males continue to express altered traits is currently unknown. Thus, males migrating from contaminated sites to relatively pristine locales have the potential to carry the phenotypic effects of EE2, and presumably the effects on mating dynamics, with them to the new site.

While our study shows that exposure to EE2 can impact mating dynamics, the effects of exposure at the population level is an equally important issue. The exact effects are difficult to predict and would be worthy subjects of future research. On the one hand, modest levels of EE2 contamination might increase the strength of sexual selection on females, because females would be competing for a smaller pool of males that would be perceived as potential mates. This increased competition for mates could reduce the effective population size, resulting in a loss of variation due to genetic drift. On the other hand, EE2 contamination could decrease the strength of sexual selection, as males made artificially unattractive by EE2 exposure may be discriminated against by attractive females, forcing them to mate with less attractive females, which would normally be excluded from the mating pool due to strong female competition. This decrease in sexual selection could lead to a reduction in population viability as mating with sub-par females could result in fewer direct or indirect benefits of mate choice and reduced offspring fitness. Overall, these considerations lead to the conclusion that the population-level effects of EE2-induced behavioral changes represent an area of critical need for future research.

Fig. 3. A male pipefish with iridescent stripes 19 days after removal from treated water. The arrow indicates the iridescent stripes, and the space between the green bars on either end of the photograph is 1.5 cm. This image shows that the feminization of males by EE2 persists after the males are moved to clean water. These traits persist at least several weeks, as they were still present when the males were sacrificed at the end of the experiment (2–3 weeks post-exposure). They may persist much longer, but this question was beyond the scope of our study.

Our study's call for additional research on population-level effects of anthropogenic contaminants that disrupt sexual selection is bolstered by other recent studies. For example, changes in reproductive hierarchies and sexual selection were observed by Coe et al. (2008) in zebrafish (*Danio rerio*) after exposure to EE2. The proportion of offspring sired by males that were dominant in the hierarchy prior to exposure was significantly suppressed after exposure to EE2, allowing subordinate males to increase their relative reproductive fitness. A similar effect was observed in the sand goby (*Pomatoschistus minutus)*, where exposure to EE2 decreased the strength of sexual selection on male size, allowing smaller males to gain mating opportunities (Saaristo et al., 2009a,b). In addition, exposure of pregnant female amarillo fish (*Girardinichthys multiradiatus*) to methyl parathion, an insecticide, suppressed the expression of secondary sexual traits in male offspring (Arellano-Aguilar and Garcia, 2008). Similar to our study, this effect resulted in the exposed male offspring being less preferred by females during choice trials. These results, along with our study suggest that solely examining the effect of these compounds on reproductive output may not be enough to determine how they are affecting populations, especially considering that processes involved in pre-copulatory sexual selection may be more sensitive than overall reproductive ability.

In summary, we found that even low levels of exposure to the endocrine disruptor EE2 in Gulf pipefish are sufficient to disrupt mating dynamics. Males exposed to EE2 become more female-like with respect to external morphology and develop secondary sexual traits that normally appear only in females. In addition, these altered traits may persist for long periods of time post-exposure. Females prefer non-exposed males compared to exposed males, and in some cases exposed males have a reduced capacity to become pregnant. Thus, EE2 exposure decreases male mating opportunities, potentially impacting their reproductive fitness. Overall, our study provides evidence for a role of endocrine disruptors in disrupting mating dynamics, a topic that remains largely unstudied. In addition, we suggest that the effects of endocrine disruptors on pre-copulatory mate choice mechanisms could have important impacts on natural populations.

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